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13. ABSTRACT ***(Maximum 200 Words)***

The research effort was directed at evaluating reagents and conditions for targeting the α-emitting radionuclide Bi-213 to breast cancer. Initial studies used human tumor xenografts (MCF-7) in athymic mice to demonstrate tumor targeting and blood/tissue clearance. Other studies evaluated placing MCF-7 cells in a renal capsule to mimic a metastatic site. Seven biotin derivatives designed to carry Bi-213 were synthesized. Four of the derivatives were radiolabeled with In-111, Y-90, and Bi-213. The In-111 labeled derivatives were tested in vivo (athymic mice) to evaluate biodistribution and to demonstrate stability of radiolabel. One of the biotin derivatives, biotin-lysine-benzyl-CHX-A'' (DTPA) appears to have properties that will allow its use in vivo. Another reagent, Bi-213 labeled succinylated streptavidin appears to be stable to in vivo demetallation, but does not appear to have a distribution that favors use in vivo. The antibody to be used in the studies, BrE3, was not provided as promised, but two other antibodies, L6 (Seattle Genetics) and NR-LU-10 (NeoRx Corp.), were kindly provided for study. Other studies included evaluation of tumor targeting with NR-LU-10-SAv conjugate or biotinylated NR-LU-10, and blood clearance with biotinylated/glycosylated BSA or Starburst™ Dendrimer. Additional studies are required to optimize the conditions for use of the new reagents.

14. SUBJECT TERMS
Breast Cancer, Radiotherapy, Alpha-Particle Therapy, Bi-213, Monoclonal Antibodies, Pretargeting, Biotin, Streptavidin

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A. INTRODUCTION

This report describes the research efforts conducted during the two year research period (August 15, 1998 thru August 14, 2000) of grant number DAMD17-98-1-8258. The research project has been focussed on developing a new approach to the therapy of metastatic breast cancer. In that development, four Objectives were outlined to accomplish that goal. The objectives were:

Objective 1: Evaluate stability and tissue distribution of ^{213}Bi-labeled biotin chelates.
Objective 2: Evaluate Biotinylated Antibody and Streptavidin Co-localization and Blood Clearance
Objective 3: Evaluate binding and toxicity of ^{213}Bi labeled biotin in an in vitro tumor cell model using the pretargeting method
Objective 4: Optimize in vivo tumor targeting of Bi-labeled biotin using the pretargeting method and determine dose delivered to tumor and normal tissues.

Research studies were conducted which addressed three of the four Objectives outlined. The reason that we did not study the last objective was that some areas of the research required more effort than originally planned, and other areas of the research were set back by this fact and by the fact that there were difficulties in obtaining the requisite materials and (MCF-7) tumor xenografts in athymic mice. Of particular note was the fact that we were unable to obtain the monoclonal antibody, BrE-3, that was promised to us prior to the start of our studies. A fair amount of effort has been spent to obtain a working antibody/tumor xenograft model that was not previously anticipated. More on this problem is provided later. These shortcomings did not allow us to complete all of tasks outlined in the Statement of Work" of the proposed studies. Instead, there were several new tasks that had to be conducted during the funding period (some of the new tasks are listed in this report as Task X1-X8). This is not unusual in exploratory research as it is not possible to predict all of the difficulties that one might encounter in such research efforts. A description of the progress made / results obtained during the funding period is provided in the following section. Areas where new information resulted in changes from the proposed studies are pointed out.

B. RESEARCH RESULTS

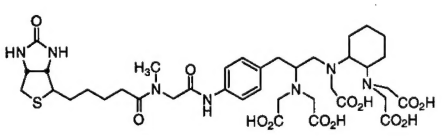
The research results are outlined below in sections based on the **Objectives**. The **Tasks** that each subheading addressed are included after the objective.

Objective 1: Evaluate stability and tissue distribution of ^{213}Bi labeled biotin chelates

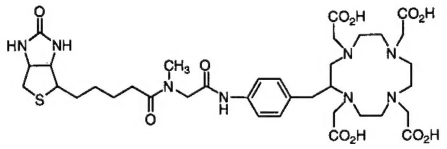
Task 1: Prepare and fully characterize biotin-sarcosine-CHX-B and biotin-sarcosine-DOTA

One approach studied for targeting Bi-213 to breast cancer is to carry it on biotin compounds. Unfortunately, biotin derivatives have several constraints to their structural designs that we (and other investigators) were unaware of when we began our studies. We have learned from other studies what those constraints are during these studies, and have adapted those designs as we have progressed in this research effort. For that reason, we synthesized and characterized seven

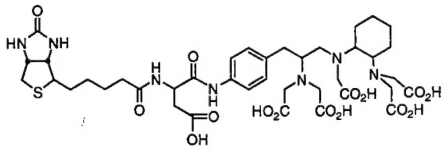
Table 1: Biotin Derivatives for Carrying Bi-211



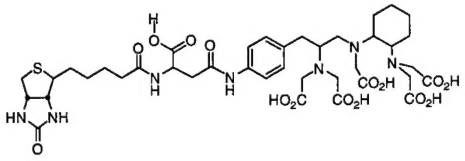
9: Biotin-Sarcosine-CHX-B



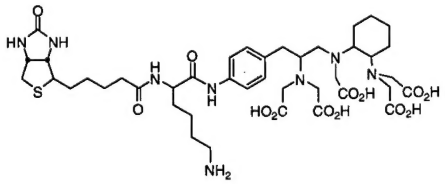
11: Biotin-Sarcosine-DOTA



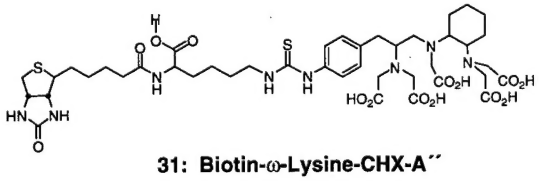
16: Biotin-α-Aspartate-CHX-A″



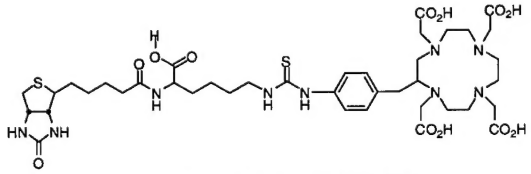
21: Biotin-β-Aspartate-CHX-A″



26: Biotin-α-Lysine-CHX-A″



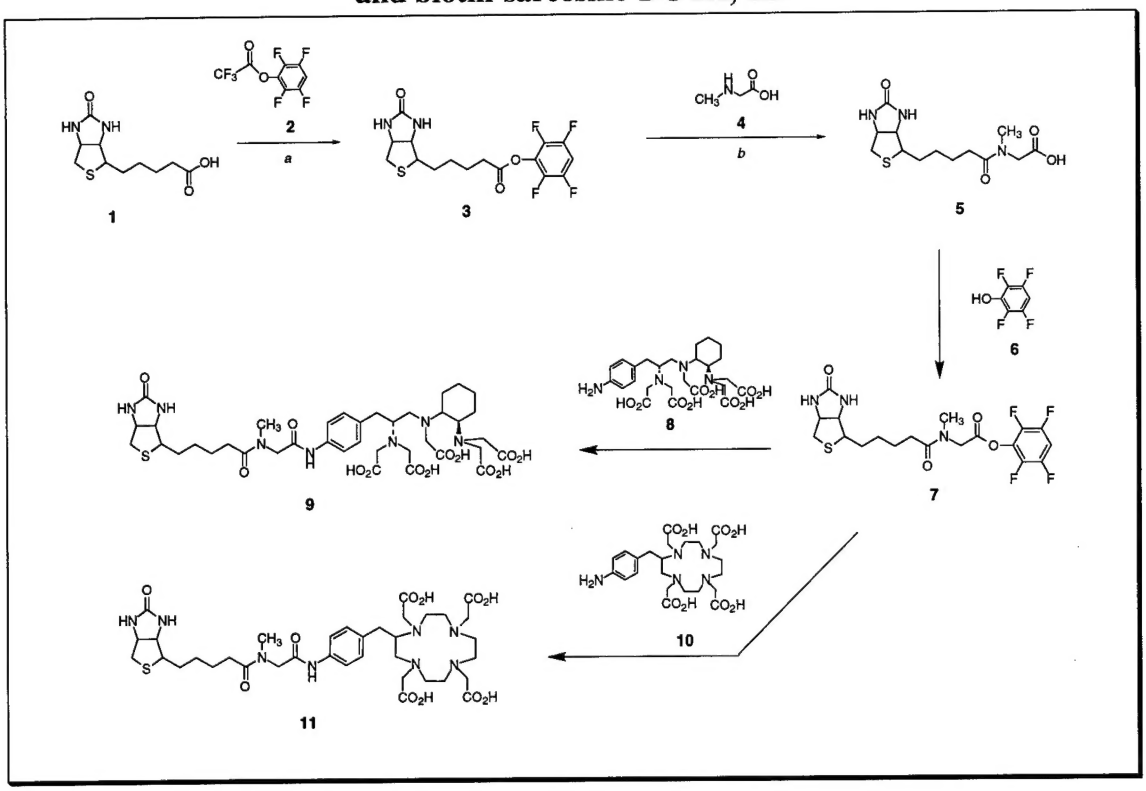
31: Biotin-ω-Lysine-CHX-A″



33: Biotin-ω-Lysine-DOTA

biotin derivatives containing chelates for ^{213}Bi during this research effort. The biotin-chelates synthesized are shown in Table 1. The first two biotin derivatives that were targeted for synthesis, biotin-sarcosine-CHX-B, **9**, and biotin-sarcosine-DOTA, **11**, were readily prepared and characterized. The synthetic pathway to obtain these two compounds is outlined in Scheme 1. Biotin-sarcosine-CHX-B, **9**, was prepared from biotin as we previously published [1], and the biotin-sarcosine-DOTA, **11**, was prepared in a similar manner. Dr. Martin Brechbiel (NIH) provided nitrobenzyl-CHX-B and nitrobenzyl-DOTA for the studies as a collaborative effort. Those compounds were easily reduced to the aminobenzyl derivatives, **8** and **10**, by catalytic hydrogenation for application to the biotin derivatives.

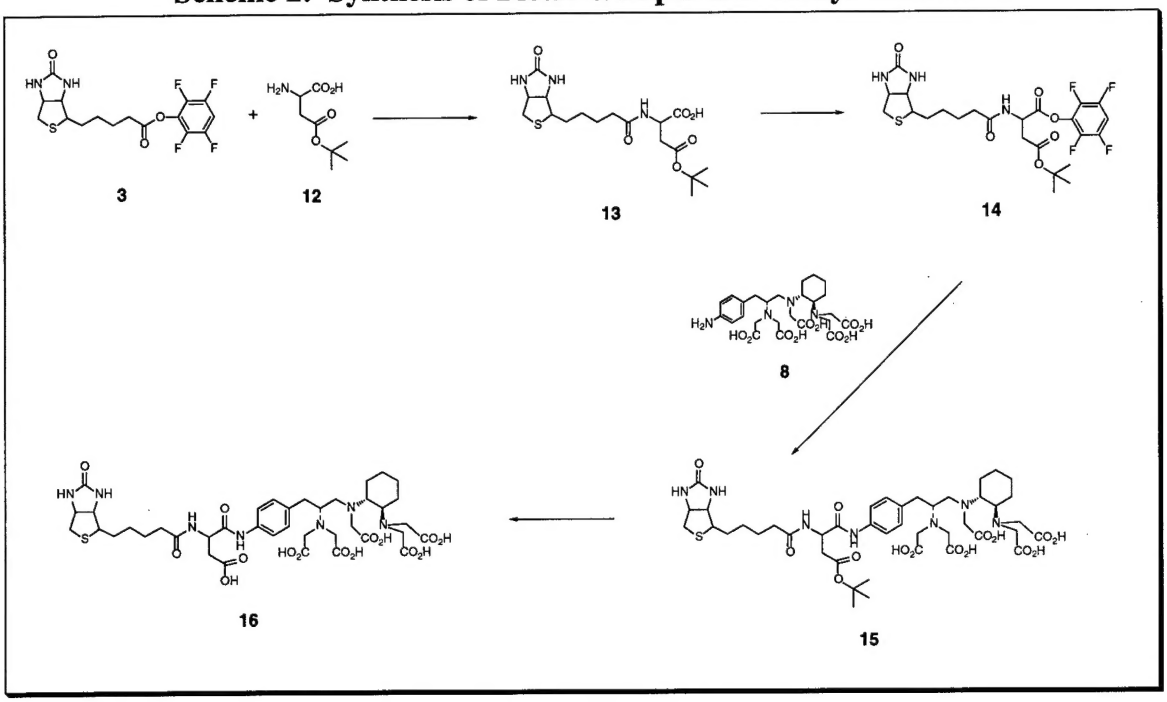
Scheme 1: Synthetic pathways to prepare biotin-sarcosine-CHX-B, 9, and biotin-sarcosine-DOTA, 11.



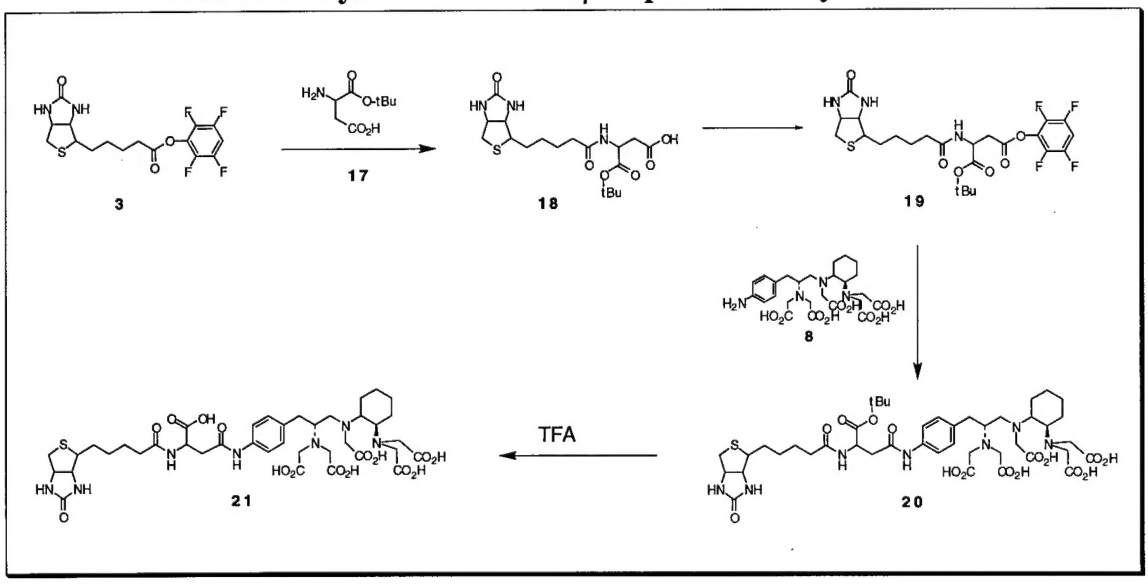
Our continuing studies with radiolabeled biotin derivatives have produced new information about what derivatives might be more favorable for in vivo use. The N-methylglycine (sarcosine) adduct of biotin was initially targeted because it was shown that this moiety effectively blocked the biotin cleaving action of the serum enzyme, biotinidase [2-4]. However, we have observed that the N-methyl group causes the dissociation of biotin from avidin and streptavidin to be greatly increased. We decided to prepare other biotin derivatives that contain the CHX-A´´ chelate for testing, so biotin derivatives that contained an aspartate moiety (**16** and **21**) were synthesized as shown in Schemes 2 and 3, and a biotin derivative containing a lysine moiety (**26**) was synthesized as shown in Scheme 4. During the period that these biotin derivatives were being prepared, we were conducting a more complete study to determine which biotin derivatives retained high binding affinities with avidin and streptavidin. In those studies (funded separately) we conducted studies to determine which (of several different) biotin derivatives retained the very slow dissociation found in unaltered biotin. We found that the length of side chain and the biotinamide moiety must be retained to obtain high binding affinities. Further, we have found that biotin derivatives with bulky substituents alpha to the biotinamide blocked biotinidase activity with only minimal increase in the dissociation rates [5,6]. With the information obtained, it was determined that biotin derivatives that contain an alpha-carboxylate, as in compound **21**, were the best for in vivo use. However, we had difficulty in the preparation of **21**, so we decided to prepare another biotin derivative, **31**, from biotin and lysine as depicted in Scheme 5. This synthesis was much higher yielding. For comparison, the biotin-lysine-DOTA derivative, **33** was synthesized in the same manner (Scheme 5). We are confident that the new biotin-lysine derivatives **31** and **32** are optimal for in vivo use, but

preparation of those compound and the biotin-sarcosine derivatives **9** and **11** has allowed direct comparison (see Task 3). All of the compounds prepared in Schemes 2 – 5 have been fully characterized.

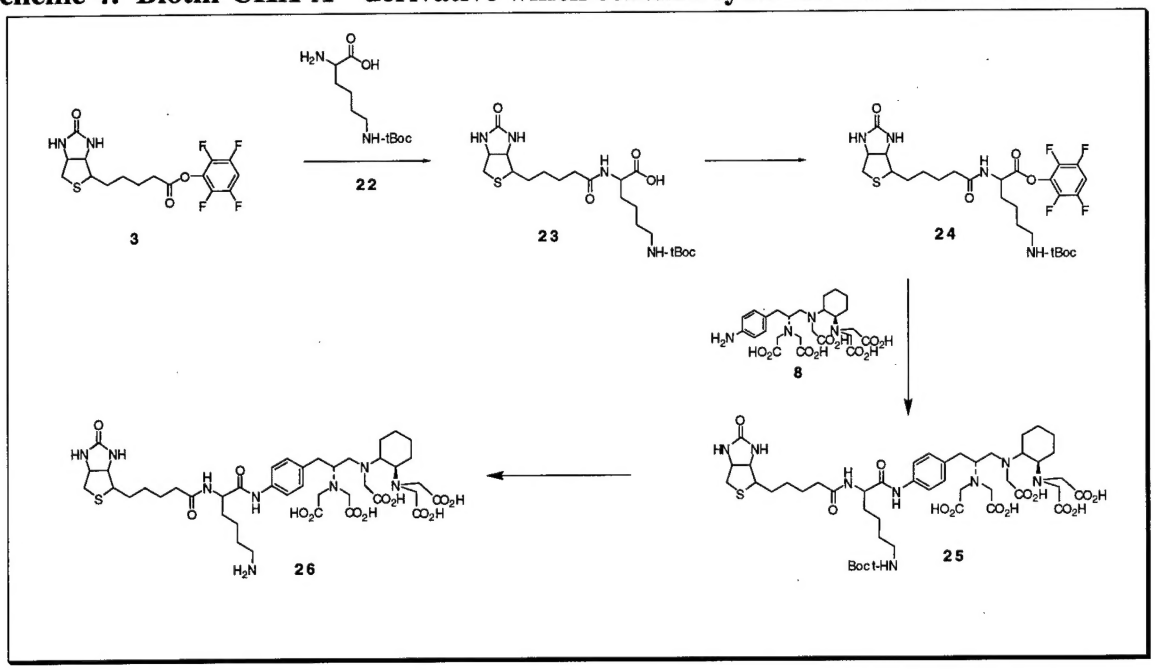
Scheme 2: Synthesis of Biotin-α-Aspartate-Benzyl-CHX-A″



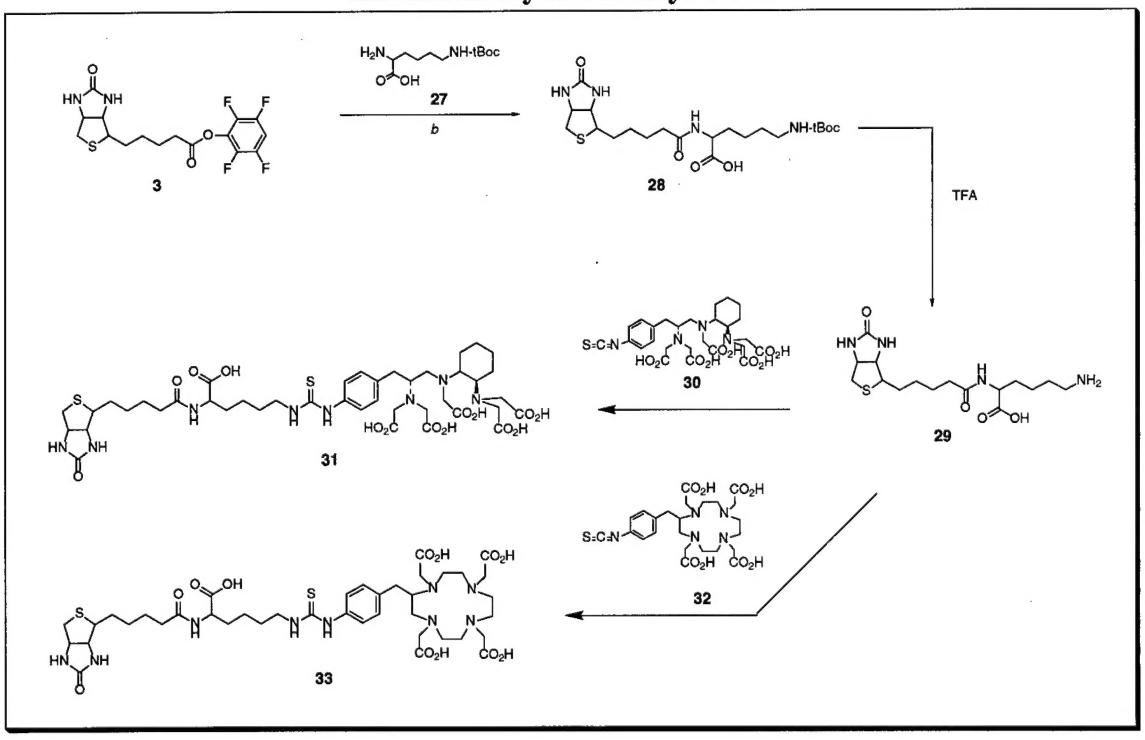
Scheme 3: Synthesis of Biotin-β-Aspartate-Benzyl-CHX-A″



Scheme 4: Biotin-CHX-A´´ derivative which contains lysine for biotinidase stabilization



Scheme 5: Synthetic pathways to prepare Biotin-Lysine-Benzyl-CHX-A´´ and Biotin-Lysine-Benzyl-DOTA



Task 2: Evaluate/optimize the Bi-213 labeling of biotin derivatives.

At the time biotin derivatives **9** and **11** were prepared, we felt that they had optimal structural features for in vivo use. Thus, we conducted further studies with them, importantly, we studied stable and radioactive bismuth labeling as depicted in Figure 1. As previously reported [1], the CHX-B derivative, **9**, labeled very quickly (almost instantaneous). Contrary to this, labeling of the biotin-DOTA derivative, **11**, with Bi was found to be slow, but it went to completion at room temperature (see chromatograms in Figure 2). Initial radiolabeling experiments with ^{213}Bi did not provide good yields.

Figure 1: Schematic representation of bismuth labeling of biotin derivatives

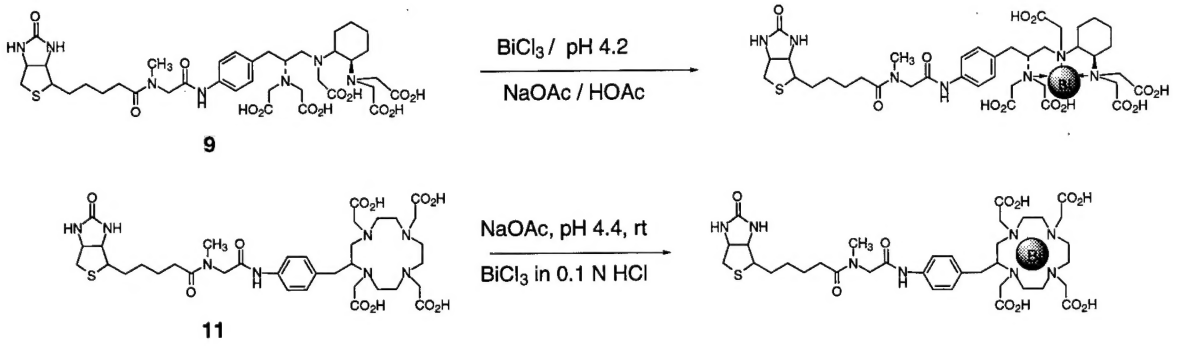
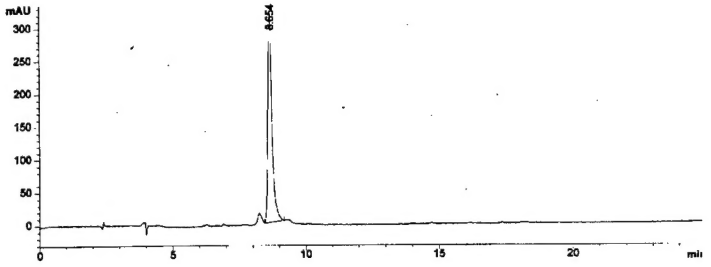
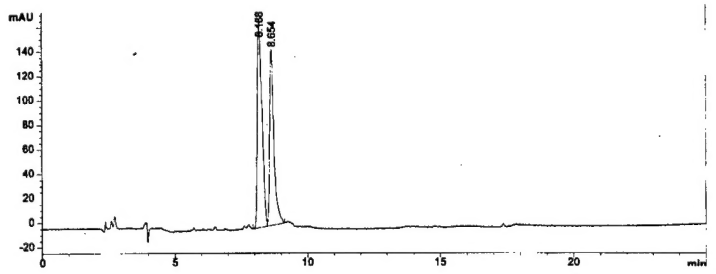


Figure 2: Scans of chromatograms from $BiCl_3$ chelation of biotin-sarcosine-DOTA

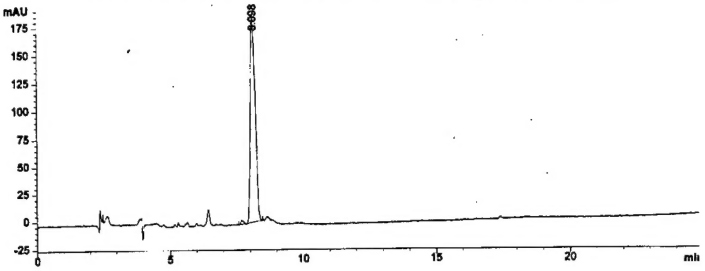
A: At 10 min reaction time – no Bi chelation



B: At 10 min reaction time – about 50% reaction



C: At 24 hour reaction time – complete chelation

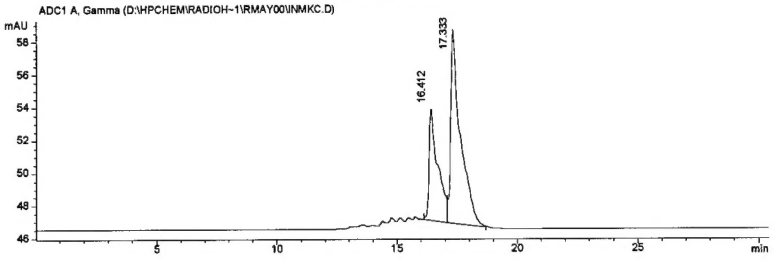


Task X1: Evaluate/optimize labeling of Biotin Derivatives with In-111 and Y-90.

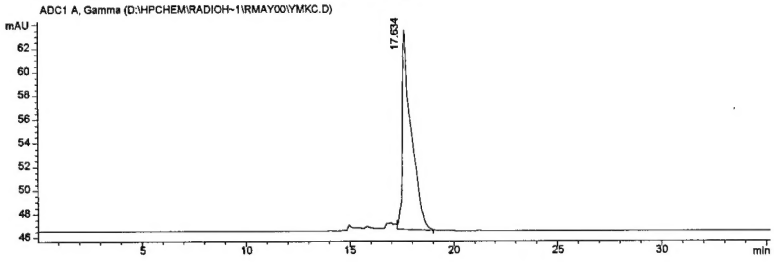
No radionuclide labelings of biotin derivatives **16**, **21** and **23** were conducted as we felt that they were not optimal, so such labeling experiments would not be fruitful. Chelation of biotin-lysine-CHX-A´´, **31**, was studied with stable indium, yttrium, and bismuth, and chelation of biotin-lysine-DOTA, **33**, was studied with stable indium. Radiolabeling of **31** was studied with the radionuclides In-111, Y-90, and Bi-213. Initial labelings were conducted with In-111 in the place of the more expensive and much shorter half-lived Bi-213 to help develop the appropriate conditions. Labeling with Y-90 was conducted as this beta-emitting radionuclide will be compared for efficacy in therapy studies with Bi-213. Bi-213 labelings were also conducted. HPLC chromatograms of the radiolabeled products obtained from reaction of **31** with In-111, Y-90 and Bi-213 conducted at room temperature for 1 hour are shown in Figure 3, panels A – C. The chromatograms were essentially the same at 10-15 min, and did not change when conducted at 37°C or 60°C indicating that all of the labeling reactions can be conducted at room temperature for 15 min.

Figure 3: Radiolabeled product from Reaction of Biotin-Lysine-CHX-A´´ and In-111 (panel A), Y-90 (panel B), and Bi-213 (panel C).

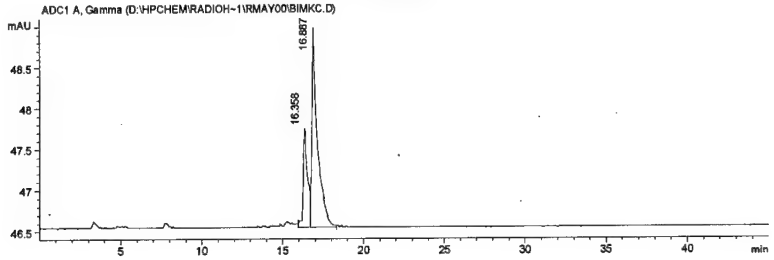
A: In-111



B: Y-90

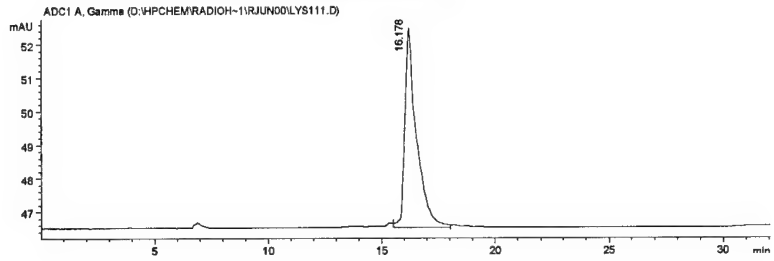


C: Bi-213



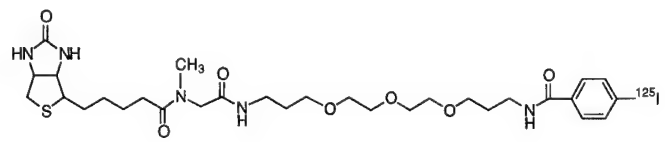
There was some concern that one of the two peaks observed for the In-111 labeling (panel A) might be an impurity, so both peaks were isolated. The isolated peaks were evaluated for binding in an avidin column and it was found that they both bound nearly quantitatively (98+%). This result leads us to believe that the two peaks observed in the HPLC chromatograms A and C represent diasteriomeric pairs. The shape of peak in chromatogram B might also indicate that there are diasteriomeric pairs present in that reaction product as well. An important aspect of the findings is that nearly quantitative labeling yields were obtained. This makes one believe that rapid labeling with Bi-213 can be achieved and it may be possible that no purification step will be required before injection. Chelation of stable indium and radiolabeling with In-111 of the biotin-lysine-DOTA compound **33**, were also evaluated. A chromatogram for that reaction product is provided in Figure 4. Additional studies with Bi-213 labeling of this compound are planned.

Figure 4: Radiolabeled product from Reaction of Biotin-Lysine-DOTA and In-111



Task 3: Evaluate distribution / in vivo stability of ^{213}Bi labeled biotin derivatives

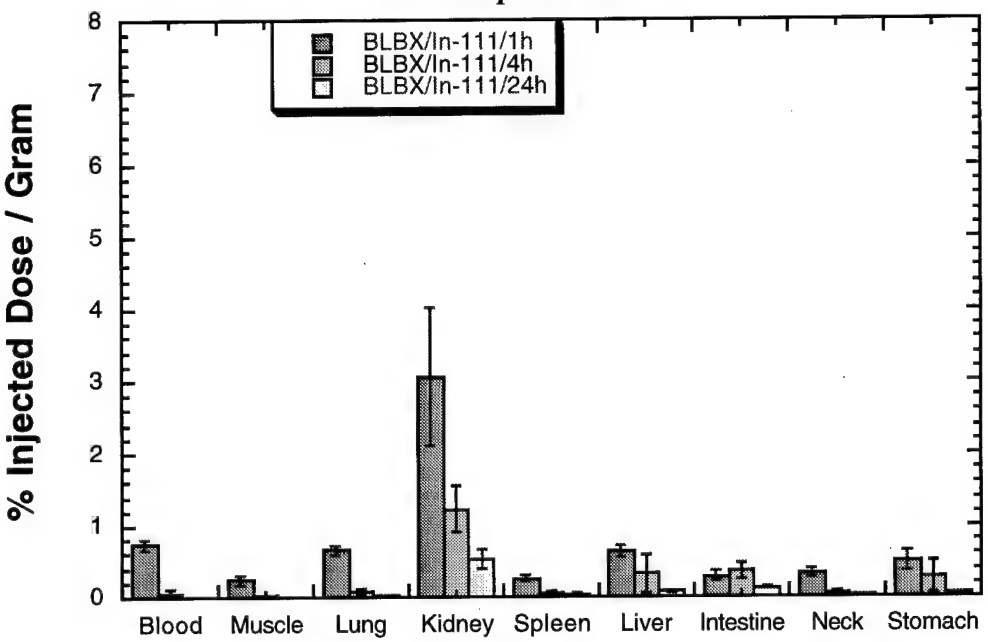
Biodistribution experiments were conducted in BALB/c nu/nu mice to determine which of the biotin derivatives; biotin-sarcosine-DOTA, **11**, biotin-lysine-CHX-A´´, **31**, and biotin-lysine-DOTA, **33**, had the most favorable in vivo distribution. The most favorable distribution would have rapid excretion by the renal system with low concentrations in other tissues, particularly liver and intestines. For this comparison, the biotin derivatives were labeled with In-111. As a control, a radioiodinated derivative of biotin, biotin-sarcosine-trioxatridecane-[^{125}I]iodobenzoate, **34**, was co-injected with the In-111 labeled biotin derivatives. The tissue distributions, which were evaluated at 1, 4 and 24 h post injection, are shown graphically in Figure 5, panels A - C.



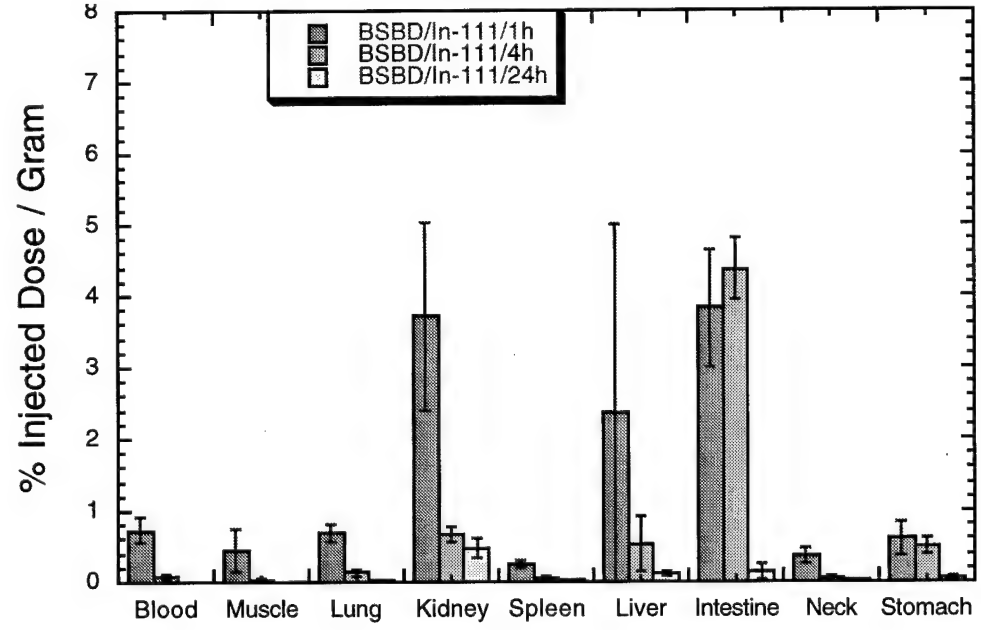
34

Figure 5: Biodistribution of In-111 Labeled Biotin Derivatives 11, 31 and 33 at 1,4,24 h pi. (Distribution of Radioiodinated 34 not shown for simplicity)

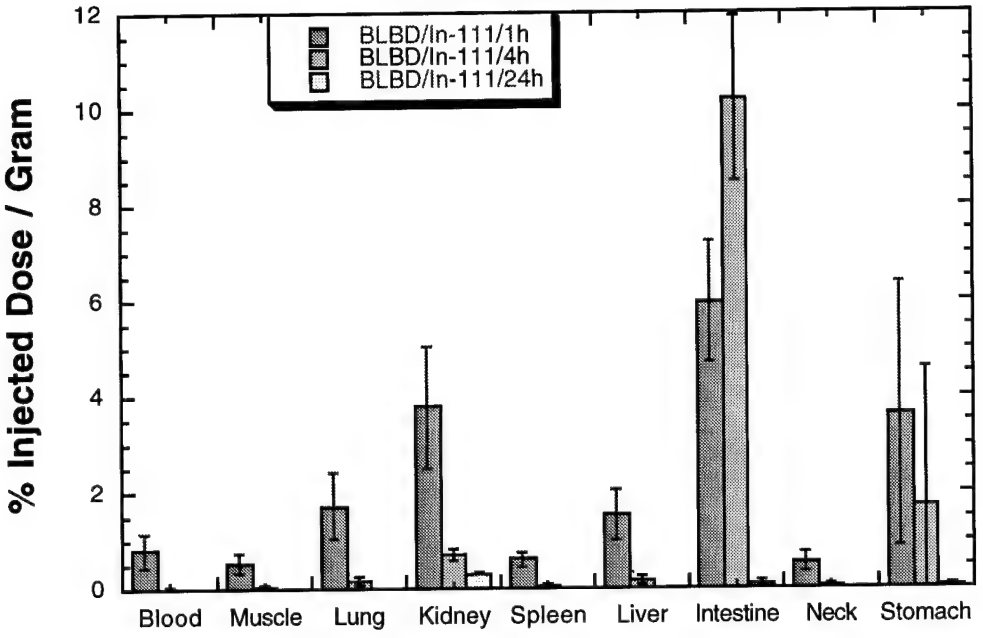
A: Compound 31



B: Compound 11

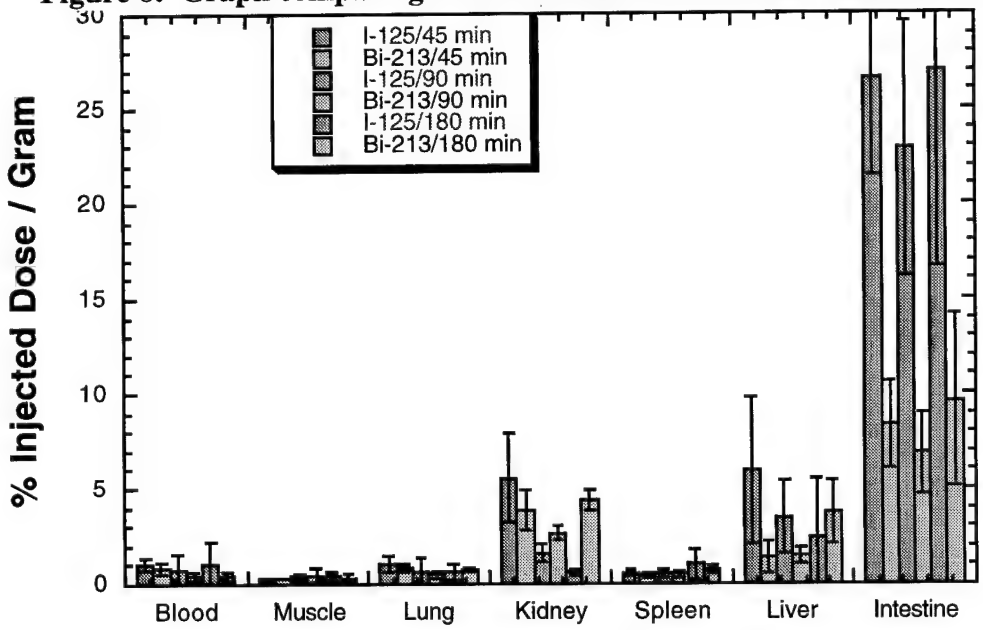


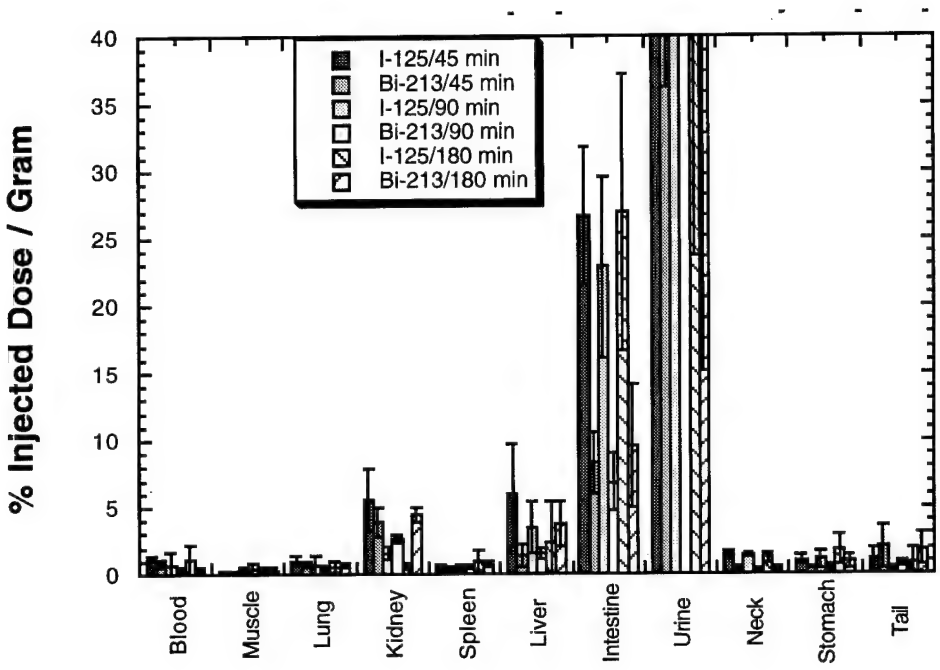
C: Compound 33



It was very clear from the biodistribution graphs in Figure 5 that the biotin-lysine-CHX-A´´, **31**, had the best biodistribution of the three biotin derivatives studies. The most important difference is that very little intestinal uptake is noted for the CHX-A´´ derivative, **31**, compared to the DOTA derivatives **11** and **33**. Another biodistribution of compound **31** was obtained after radiolabeling with Bi-213. The data obtained are presented graphically in Figure 6. The distribution of radionuclide in vivo was examined at 45 min, 90 min and 180 min. These

Figure 6: Graph comparing Bi-213 Labeled 31 and I-125 Labeled 34





timepoints roughly represent 1, 2 and 4 half-lives of Bi-213. An important aspect of the study was to determine if the Bi-213 is stably attached to the CHX-A´´ (DTPA) chelate. Previous animal studies had shown radionuclides bound to the CHX DTPA were quite stable in vivo [7,8], so we expected the Bi-213 labeled biotin-lysine-CHX-A´´ to also be stable. Again, the radioiodinated biotin derivative **34** was co-injected as a control. The relative amounts of radionuclide (I-125 vs Bi-213) in the kidney should be noted. The fact that there is more Bi-213 than I-125 in the kidney may indicate that some of the Bi-213 is released from **31**. This result suggests that additional studies should be pursued to determine if the Bi-213 is actually being released. If it is, additional studies will be required to prepare a more stable Bi-213 derivative. A study to determine if the biotin-lysine-DOTA, **33**, holds Bi-213 more stably is planned.

Objective 2: Evaluate Biotinylated Antibody and Streptavidin Co-Localization and Blood Clearance

Task 4: Obtain humanized BrE-3 from Dr. Ceriani (Walnut Creek, CA)

The original proposal provided little effort for obtaining an anti-breast antibody. This was the case as I received a letter from Dr. Roberto L. Ceriani of the Cancer Research Institute of Contra Costa that indicated up to 100 mg of huBrE-3 would be provided for the study (letter attached as Appendix Item 1). Early in the studies I wrote a letter to Dr. Ceriani to make arrangements to obtain the antibody. I received no response, but I felt that he was a busy person so I did not aggressively pursue it. My timeframe for getting the antibody was at 6 months into the project as it was not needed until then. As that time approached, I called his office, sent several e-mails, and wrote another letter. I have obtained no response. I have tried many times since then to reach him via e-mail (internet http://mypage.ihost.com/CancerResearchInstitute) and by phone

(925-943-1167), but have not spoken with him directly and have not received any responses thus far. (This has been the most frustrating situation that I have ever run up against!).

Task X2: Obtain alternate antibody for studies.

After it was clear the huBrE-3 would not be obtained, I began to look for another anti-breast cancer antibody. I spoke with Dr. Alan Fritzberg at NeoRx Corporation (Seattle) about breast cancer antibodies and he indicated that their pan-carcinoma antibody NR-LU-10 had detected breast cancers. He indicated that they would collaborate by providing the antibody, but that a material transfer agreement had to be put in place between NeoRx and the University of Washington. The paperwork was put together and sent to our Office of Technology Transfer OTT). The OTT found the wording of the material transfer agreement to not be acceptable and NeoRx would not change the wording. Therefore, I could not obtain the antibody from them. I looked for another breast cancer antibody and decided that the L6 antibody that was in several publications of Dr. DeNardo [9-11] might be a good candidate for our studies. This antibody was owned by Bristol-Myers Squibb, but recently was acquired by a new startup company in Seattle, Seattle Genetics. Dr. Perry Fell of Seattle Genetics agreed to provide the antibody for our studies, and we were successful in getting a material transfer agreement in place. They kindly provided 100 mg of L6 for the studies.

Task 5: Begin to grow up MCF-7 cells for use in single cell evaluations

MCF-7 cells were obtained and grown up for use in Dr. Vessella's laboratory for use in these studies.

Task 6: Biotinylate the antibody and evaluate the number of biotins on it.

The L6 that obtained was biotinylated and assessed for the number of biotins present. It was found that there were 1.3 biotins / antibody by the HABA test. Before this parameter was optimized, it was important to test the cell binding of biotinylated L6 with MCF-7 cells vs non-biotinylated L6. Biotinylation of NR-LU-10 was conducted after it was found that antibody would be used in the studies. The biotinylation of NR-LU-10 was conducted using 8 equivalents of biotin-LC-NHS ester to give 4 biotins per antibody..

Task 7: Radioiodinate biotinylated L6 and evaluate binding with MCF-7 cells.

Cell immunoassays were conducted on aliquots of 1 x 10^6 cells, with 2 assays / sample. The cells were either fresh or thawed and adjusted to 1 x 10^6 viable cells if revived from frozen aliquots. After addition of radiolabeled L6 or radioiodinated/biotinylated L6, the cells were incubated at 37°C for 45 min on a cell rotator. In the first experiment, the amount of L6 required to saturate the MCF-7 cells was examined. To obtain this, increasing quantities of radioiodinated L6 were added to fresh MCF-7 cells in an attempt to determine when the antibody was in excess. It was found that the radioiodinated L6 bound only to the extent of 2.6%. This result was troubling, so another experiment was conducted where a non-specific radioiodinated antibody, MOPC-21 was compared with radioiodinated L6 and radioiodinated/biotinylated L6. The results

were not good. The MOPC-21 bound 4.6-5.3 %, the L6 bound 5.2-6.8 % and the L6-biotin bound 2.8-3.6 %. This meant that there was no binding of L6 to MCF-7 cells.

The previous result prompted us to test the radiolabeled L6 against a number of other breast cancer cell lines (ductal and epithelial lines – all cells are in tissue or cell culture bank at ATCC). The cell lines tested were:

Cell Line	Binding
SK-BR-3 (passage 3, Vessella),	2.6%
BT-20 (passage 292),	4.2%
T47D (passage 91),	2.6%
ZR-75-1 (passage 1, Vessella)	2.8%
MDB0M8-134VI (passage 83)	4.9%

The results clearly indicate that there is no specific binding of the radioiodinated L6 to the breast cancer cells tested. We have contacted Seattle Genetics and asked them whether the antibody batch that they gave to us might be bad (it has been stored for several years) and whether there might be another breast cancer cell line that it binds with. We have not gotten a reply yet.

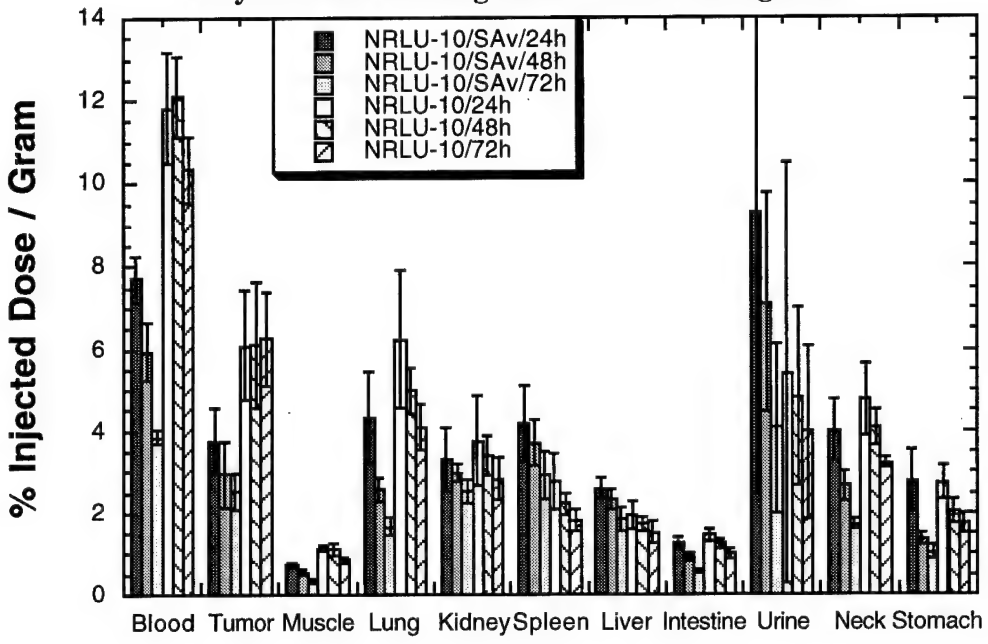
With the prospect that the L6 may not be a good candidate antibody, we went back to NeoRx Corporation and opened discussions again about obtaining NR-LU-10 for our studies. They allowed the University of Washington to provide a material transfer agreement and signed it. We obtained NR-LU-10 and have been using it in the place of BrE3 in the studies.

Task X3-X5: Prepare NR-LU-10-SAv Conjugate, Radioiodinate and Evaluate Tumor Localization.

These tasks were not in the original proposal, but were required for our studies. Task X3, conjugation of NR-LU-10 and rSAv, took a great deal of time. Conditions for the conjugation and purification steps were worked out and an overall yield of approximately 30% purified conjugate was obtained. Task X4, radioiodination of NR-LU-10-SAv conjugate was readily achieved.

A biodistribution (Task X5) comparing [^{125}I]NR-LU-10-SAv conjugate with [^{131}I]NR-LU-10 was conducted in athymic mice bearing MCF-7 human breast cancer xenografts. Tissue distributions were obtained at 24 h, 48 h and 72 h post injection. The data obtained is shown in Figure 7. It can be noted that the NR-LU-10-SAv conjugate cleared much more rapidly than the NR-LU-10. This resulted in the NR-LU-10 having lower blood, tumor and lung concentrations at all timepoints. However, higher spleen and liver concentrations were noted for the NR-LU-10-SAv conjugate, perhaps indicating that some of the conjugate was aggregated. In general, the tissue/blood ratios were nearly the same so we felt that this conjugate was appropriate for further studies.

Figure 7: Comparison of the Biodistributions of NR-LU-10-SAv and NR-LU-10 in Athymic Mice Bearing MCF-7 tumor xenografts



Task 8: Prepared a biotinylated blood clearance reagent and characterize. Demonstrate binding with streptavidin.

Although it was not in our original research plan, we decided to investigate three different clearing agents to determine which was best for this application. Thus, we prepared biotinylated asialoorosomucoid protein [12] and biotinylated and glycosylated bovine serum albumin (BSA) and the Starburst Dendrimer generation 2. Evaluation of the clearing capabilities of two of these agents was tested (see below).

Task 9: Evaluate blood clearance of radiolabeled antibody-streptavidin conjugate using the biotinylated clearing agent.

Biotinylated and glycosylated BSA and Starburst™ Dendrimer generation 2 were tested as agents for clearance of NR-LU-10-SAv from blood. In the first group, athymic mice bearing MCF-7 tumor xenografts were injected with 300 μg of NR-LU-10-SAv and after 24 h, 100 μg of biotinylated and glycosylated Starburst Dendrimer (BSBD) was injected. Biodistributions were evaluated 2 h after injection of the clearing agent. The biodistribution is shown in Figure 8. It is very apparent that no clearance was obtained with this reagent.

In the second and third groups, athymic mice bearing MCF-7 tumor xenografts were injected with 300 μg of NR-LU-10-SAv and after 24 h, 100 μg (group 2) or 200 μg (group 3) of biotinylated and glycosylated BSA (BBSA) was injected. The results are shown graphically in Figure 9. As a control, NR-LU-10 without the SAv present was subjected to the same clearing conditions. Those results are shown in Figure 10. It is apparent that rapid clearance of the NR-LU-10 from blood was obtained (in approx. 70% decrease) using the modified BSA. That is the

reagent that we use routinely now. It appears that further studies of clearing agents are required to obtain optimal clearance.

Figure 8: Clearance of NR-LU-10-SAv Conjugate with Biotinylated and Glycosylated Starburst™ Dendrimer

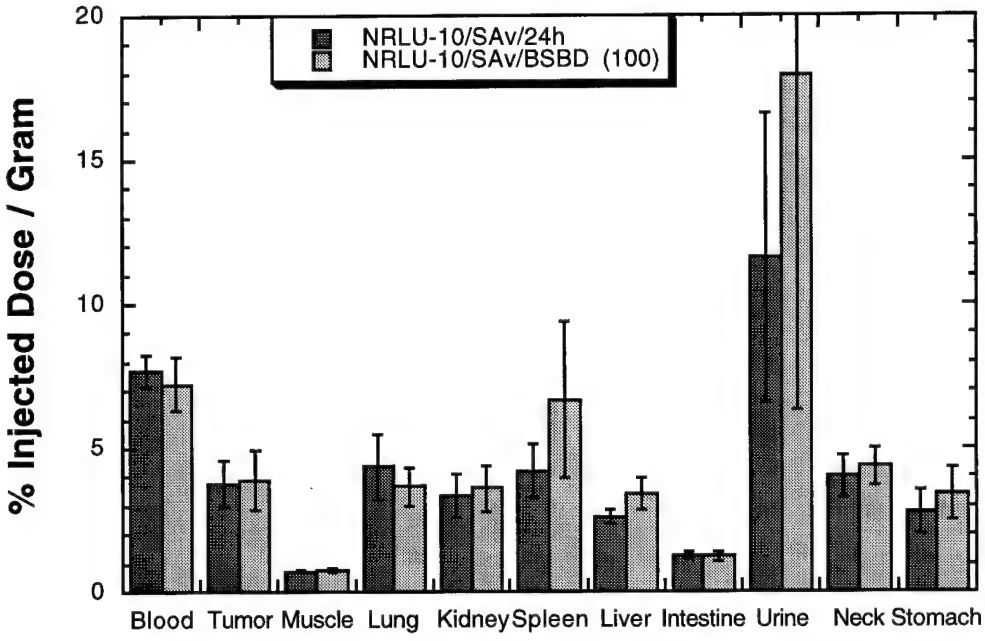


Figure 9: Clearance of NR-LU-10-SAv Conjugate with Varying Quantities of Biotinylated and Glycosylated BSA

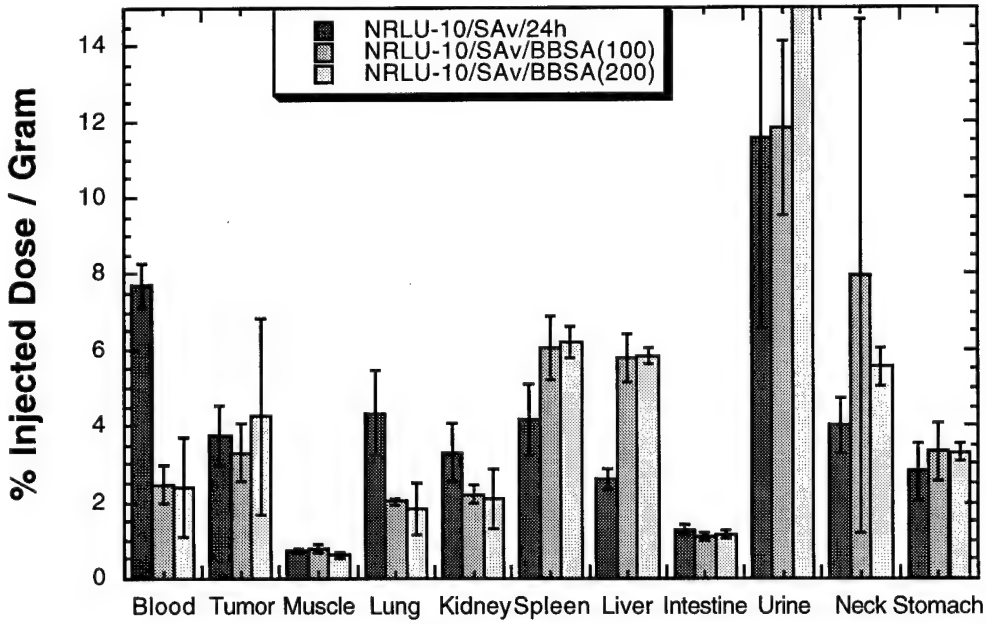
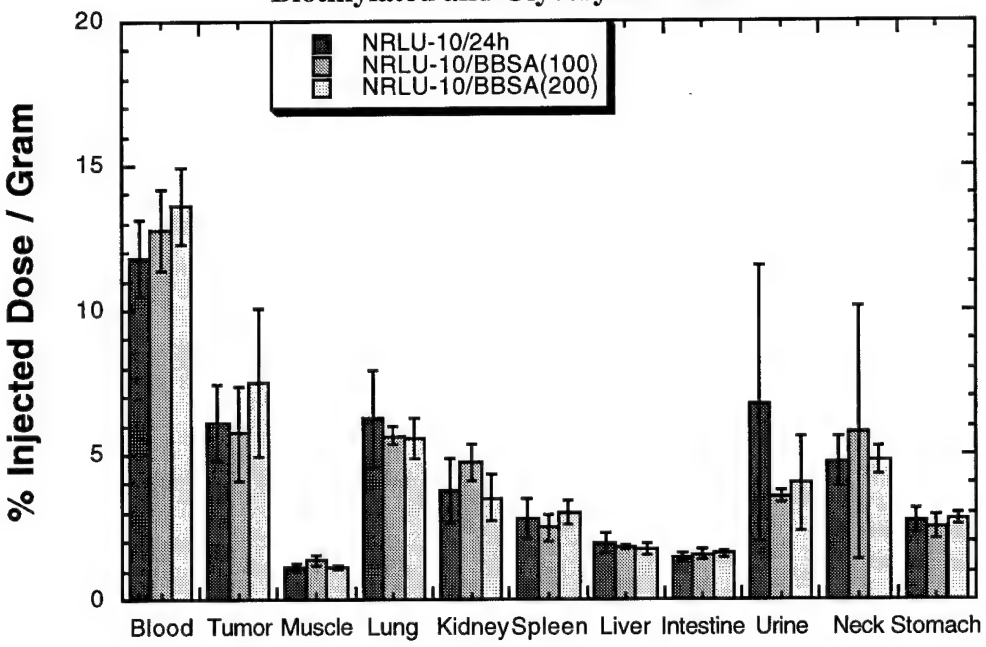


Figure 10: Clearance of NR-LU-10 with Varying Quantities of Biotinylated and Glycosylated BSA



Task 10: Place cells in subrenal capsule for use in tumor localization studies.

Studies were conducted to determine if MCF-7 would grow in subrenal capsules, the metastatic model that we are going to use in these studies. Twenty animals were injected with 2 x 10^5 cells under the renal capsule. Of those injected, 15 animals grew observable tumors (a 75% take rate). The animals (groups of 4) were assigned a time for sacrifice to assess tumor burden (at 2,3,4,5, and 6 weeks post implantation). The tumor burdens were assessed via three methods.

1. Tumor volume (ovoid mass in 3 dimensions). This was calculated by the formula: length x width x height x 0.5236, which is the most common formula for subcutaneous tumors.
2. Change in kidney mass. This measurement is based on the weight of the implanted kidney vs non-tumor bearing kidney. The problem with this measurement is that there may be a lot of apoptosis occurring and the kidney can decrease in weight rather than increase.
3. Surface area of lesion (length x width). This seemed to be the most accurate as depth was difficult to judge due to infiltration of the cells into the cortex, whereas the surface of the capsule was easy to measure.

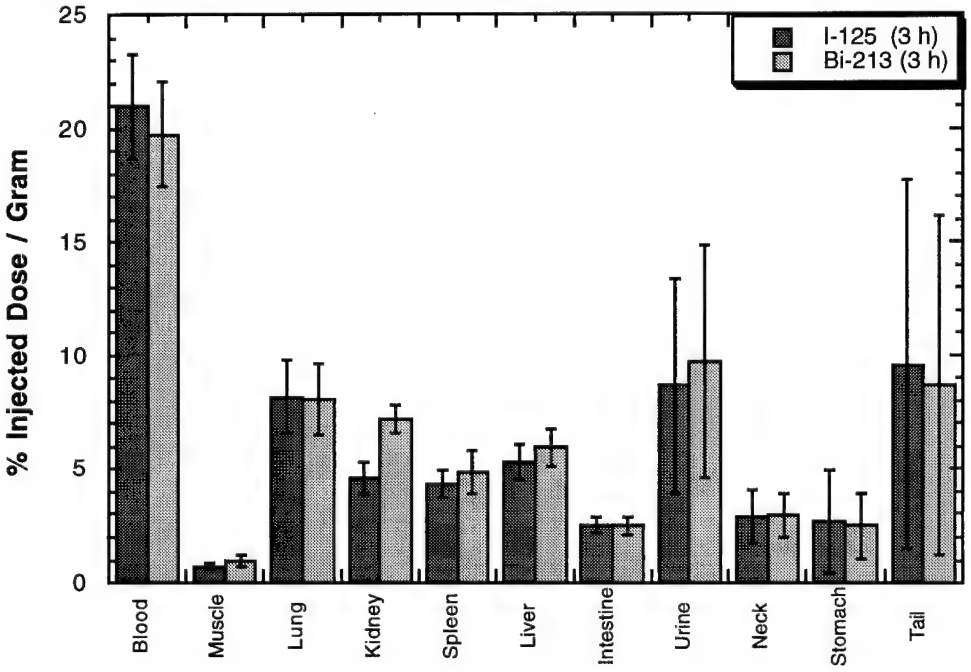
A graphical representation of results obtained using the three methods of measurement out above is included as Appendix Item 2. The graphs show that there is no clear trend in size of tumor with time from implantation.

Task X6: Determine stability of ^{213}Bi-Labeled Succinylated rSAv

Our original proposal did not include this task. However, we were interested in the possibility of using a 2-step pretargeting protocol that has the ^{213}Bi attached to streptavidin (targeting biotinylated antibody) rather than the 2-step protocol where ^{213}Bi is on a biotin derivative (targeting the antibody-streptavidin conjugate) so we conducted an animal study with ^{213}Bi-labeled succinylated streptavidin vs radioiodinated chelated streptavidin. Succinylated streptavidin has been shown to not localize to kidney as the native streptavidin does [13,14]. For such a pretargeting system to work, it was important that the ^{213}Bi be stably attached to the succinylated rSAv. Therefore, the stability was assessed in an animal study.

In the study, streptavidin was demetallated, conjugated with isothiocyanato-benzyl-CHX-A´´, and succinylated. It was again demetallated and stored for use. A quantity of this material was labeled with ^{125}I and another quantity was labeled with Bi-213. The radiolabeled succinylated/CHX-A´´ conjugated streptavidin was injected into athymic mice that did not have tumor xenografts. Mice were sacrificed at 45 min, 1.5 h, and 3 h after injection. The times were chosen as they represent 1, 2, and 4 half-lives of Bi-213. A bar graph of the data obtained at the longest time point (3h) is provided in Figure 11. The primary goal of this experiment was to determine how stable the ^{213}Bi label was on streptavidin over the period of 4 half-lives. It is apparent from the data that the ^{213}Bi is relatively stable on the succinylated streptavidin as only a small difference is observed in the kidney after 4 half-lives (earlier times had smaller difference in kidney concentration of radionuclides. This experiment indicates that it may be possible to use succinylated streptavidin as the carrier of ^{213}Bi if the cancer cells are readily accessible to the blood, which may be the case for metastatic cancer cells.

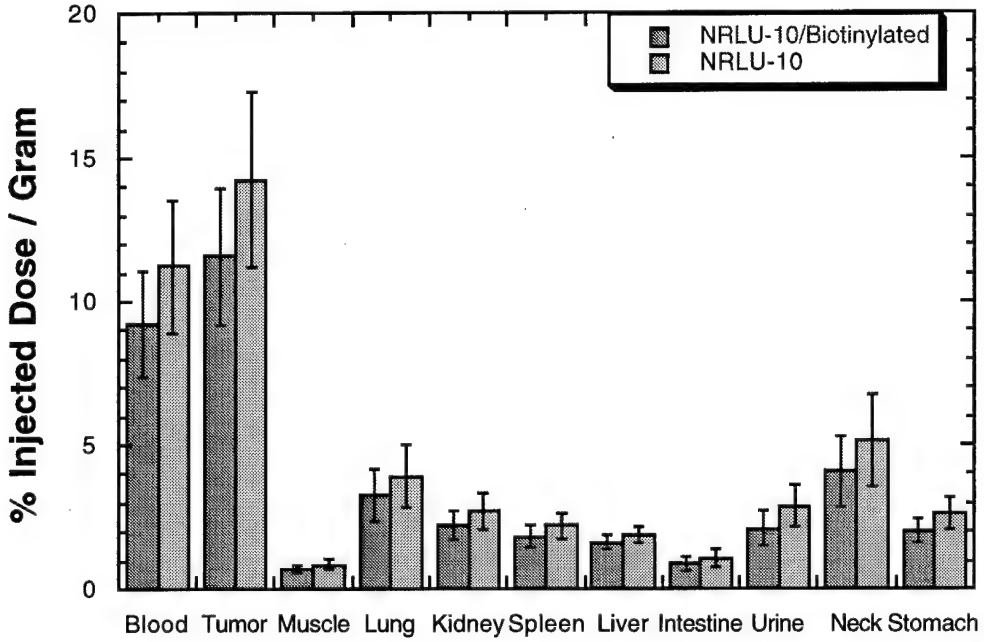
Figure 11: Biodistribution of ^{213}Bi- and ^{125}I-labeled Succinylated Streptavidin at 3 h pi



Task X7: Comparison of the Distributions of Biotinylated NR-LU-10 and NR-LU-10

As the NR-LU-10-SAv conjugate appeared quite different from unmodified NR-LU-10, we felt it was important to include a study to determine if biotinylated NR-LU-10 had a distribution similar to that of unmodified NR-LU-10. That study was conducted by co-injecting 25 μg of each of biotinylated [^{125}I]NR-LU-10 and [^{131}I]NR-LU-10 in athymic mice bearing MCF-7 tumor xenografts. The distributions, obtained at 48 h pi, are shown graphically in Figure 12. It is apparent that the two species have very similar distributions.

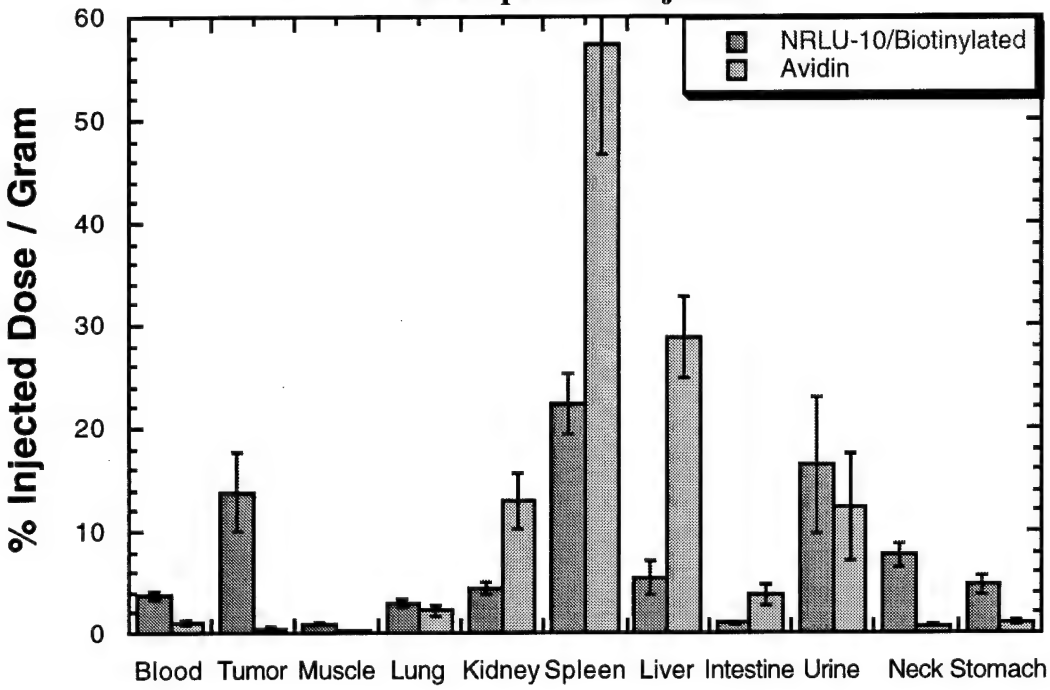
Figure 12: Comparison of Biotinylated NR-LU-10 and NR-LU-10 in Athymic Mice bearing MCF-7 Tumor Xenografts



Task X8: Evaluation of the Clearance of Biotinylated NR-LU-10 with Avidin.

A study was conducted to determine the effectiveness of clearing biotinylated NR-LU-10 from blood with avidin. In the experiment, athymic mice bearing MCF-7 tumor xenografts were injected with 50 μg of biotinylated [^{125}I]NR-LU-10 and after 48 h, 25 μg of [^{131}I] avidin was injected. Distributions of the two radiolabeled species were examined 4 h pi of the avidin. The results are shown graphically in Figure 13. If one compares the results shown in Figure 12 (no avidin at 48h) with that in Figure 13 (note differences in scales), it can be observed that the biotinylated NR-LU-10 is cleared from blood but the tumor localization remains the same. The high spleen concentration of biotinylated NR-LU-10 are readily explained by the clearance of avidin in that organ. High neck and stomach concentrations area due to degradation of the radioiodinated antibody with release of radioiodide. These results indicate that avidin will work well as a clearing agent in this system.

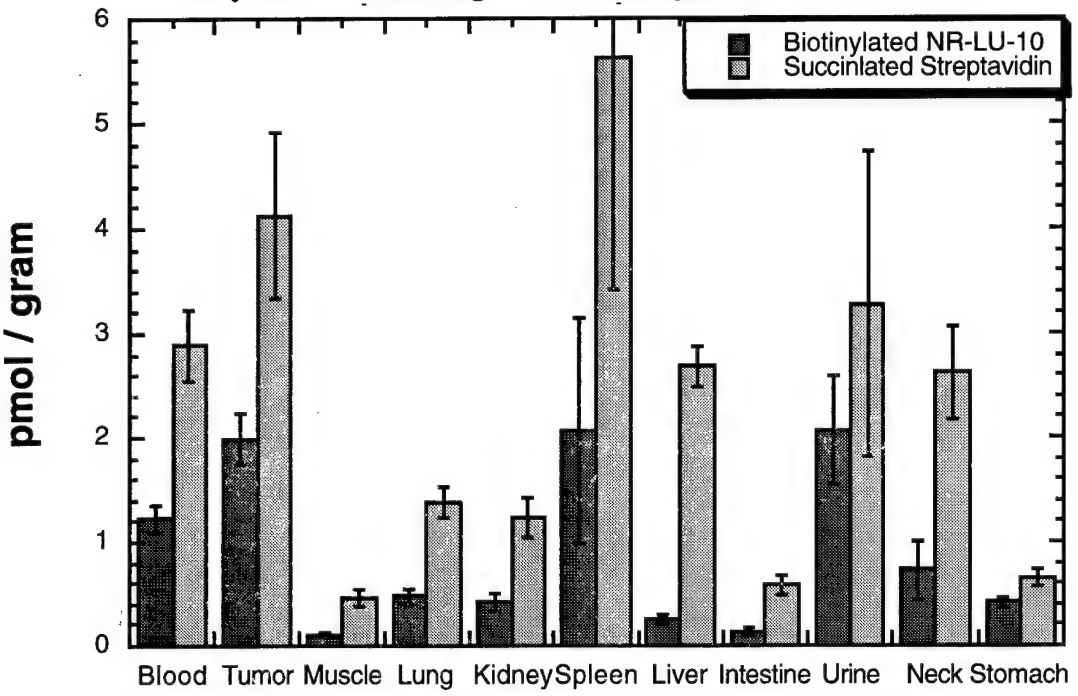
Figure 13: Distributions of Biotinylated [^{125}I]NR-LU-10 and [^{131}I]Avidin at 4 h pi Avidin Injection



Task 11: Evaluate Tumor Localization of biotinylated antibody and Co-localization with Streptavidin.

In this study, 50 µg of biotinylated [^{125}I]NR-LU-10 was injected into mice bearing MCF-7 tumor xenografts. After 48 h, 25 µg of avidin was administered, and an additional 4 h, succinylated [^{131}I]rSAv was administered. The biodistribution of radiolabeled species was determined at 24 h pi SAv injection (72h pi NR-LU-10). The biodistribution data is shown graphically in Figure 14. Because the rSAv was not cleared from blood, its concentration is high in all tissues. Importantly, it appears to localize well to the tumor. Liver, spleen and neck (thyroid) concentrations appear to be higher than expected from just blood volume, so some non-specific localization in these tissues is probably occurring. These data are interesting, but put into question whether Bi-213 could be used on succinylated rSAv as a means of targeting breast cancer cells in vivo. We believe that this might work only for metastatic cells in blood and spleen.

Figure 14: Biodistribution of Biotinylated [^{125}I]NR-LU-10 and Succinylated [^{131}I]SAv in Athymic Mice Bearing MCF-7 Xenografts (24h pi SAv)



Objective 3: Evaluate binding and toxicity of ^{213}Bi-labeled biotin in an in vitro tumor cell model using the pretargeting method.

Tasks 12: Evaluate cell survival of MCF-7 cells in culture when treated with biotinylated antibody / streptavidin and biotinylated antibody and ^{213}Bi-labeled biotin.

We have conducted studies with another antibody CA12.10 (a canine anti-CD45 antibody reactive with hematopoietic cells) to assess cell survival with Bi-213 labeled antibodies. In those studies we demonstrated that the antibody directly labeled with ^{213}Bi provided very efficient cell killing when labeled at high specific activity (RBE about 10). At low specific activity, cell survival was the same as that found for a non-specific labeled antibody. With saturation of the cells, all of the cells were killed. The results were compared with a dose-equivalent response of external beam gamma irradiation. The data obtained is provided as Appendix Item 3.

Task 13: Continue cell survival studies with Bi-labeled biotin

This task was not conducted as we felt that it was not necessary based on previous results obtained (i.e. Bi-213 killed all of the cells that it bound).

Task 14: Calculate doses delivered and examine the biological effectiveness of the doses provided by ^{213}Bi.

A portion of this task has been done as described above. Based on literature findings and other aspects of these studies, we did not feel that additional information was required.

Objective 4: Optimize in vivo tumor targeting of Bi-labeled biotin using the pretargeting method and determine dose to tumor and normal cells.

Our studies have not progressed to the point of optimizing the reagents required. At this time we do not believe that Bi-213 labeled succinylated streptavidin is a viable method for targeting cancer cells for killing. This fact makes completion of tasks 15 and 16 not necessary. In

Tasks 15: Evaluate biodistribution of varying quantities (e.g. 5, 25, 50 mg) of biotinylated [^{125}I]antibody, and the biodistribution of co-localized [^{131}I]streptavidin.

Not required.

Tasks 16: Evaluate biodistribution of optimized quantities of biotinylated [^{125}I]antibody and [^{131}I]streptavidin.

Not required.

Tasks 17: Evaluate co-localization of biotinylated [^{125}I]antibody, streptavidin and [^{213}Bi]biotin.

Not completed.

Tasks 18: Evaluate biodistribution of biotinylated [^{125}I]antibody, streptavidin and [^{213}Bi]biotin (at 3 different specific activities).

Not completed.

Tasks 19: Evaluate biodistribution of biotinylated [^{125}I]antibody, streptavidin and [^{213}Bi]biotin under optimal conditions.

Not completed.

Task 20: Evaluate doses delivered to normal tissues and MCF-7 cells in renal capsule.

Not completed.

C. KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized and characterized five biotin-CHX-A´´ derivatives and two biotin-DOTA derivative for labeling with ^{213}Bi.
2. Tested all of the biotin derivatives for labeling with In-111 and some with Y-90 and ^{213}Bi.

3. Evaluated in vivo characteristics of radiolabeled biotin derivatives.
4. Obtained MCF-7 cells, grew them in culture, and implanted in subrenal capsules for the animal model to be used. Had difficulty in getting the MCF-7 tumor xenografts to grow consistently.
5. Attempted to obtain huBrE-3 antibody from Dr. Ceriani (did not get response). Obtained L6 antibody from Seattle Genetics for testing. Due to low binding characteristics of this antibody, we did not use it. Obtained NR-LU-10 antibody from NeoRx Corp. and used in studies.
6. Successfully succinylated streptavidin and evaluated its in vitro and in vivo characteristics with various levels of succinylation (done in concert with other studies).
7. Biotinylated and radioiodinated L6. Found that it did not bind with several different breast cancer cell lines. Biotinylated NR-LU-10 and used in studies.
8. Prepared and characterized biotinylated asialoorosomucoid protein, biotinylated/glycosylated bovine serum albumin (BSA) and biotinylated/glycosylated StarburstTM Dendrimer generation 2. These three clearing agents were tested for clearing streptavidin and/or antibody-streptavidin conjugates from blood. The biotinylated/glycosylated BSA worked well.
9. Determined that the ^{213}Bi-label is stably attached to streptavidin over three half-lives in mice. However, the distribution makes it questionable whether this reagent can be used in the pretargeting regimens.
10. Determined that ^{213}Bi-labeled antibody is very cytotoxic with a RBE of approximately 10 when it is used at a very high specific activity.

D. REPORTABLE OUTCOMES:

There have been several presentations that included data from these studies and one manuscript is being prepared for submission to a journal. A list of the presentations made and the manuscript in preparation follow.

Wilbur D.S. (1998) Radiolabeling and Targeting of Alpha-Emitting Radionuclides for Radiation Therapy. Presented (invited) at the Northwest Regional American Chemical Society meeting, Pasco, Washington, June 17-20, 1998.

Wilbur D.S. (1998) Development of Reagents for Application in the Pretargeting Approach to Targeted Radiotherapy. (Invited) Presented at Uppsala University, Uppsala, Sweden, October 22, 1998.

Wilbur D.S., Pathare P.M., Hamlin D.K., Vessella R.L., Buhler K.R., Stayton P.S., and Hyre D.E. (1999) Development of Biotin Conjugates for Antibody-Based Targeted Radiotherapy. Presented (invited) at the 217th Annual Spring Meeting of the American Chemical Society in Anaheim, CA, March 21-25, 1999.

Wilbur D.S. (2000) An Overview of the Design of Biotin Reagents for In Vivo Application. (Invited) Presented at The *First International Conference on (Strept)Avidin-Biotin Technologies*, held June 18-21, 2000 in Banff, Alberta, Canada. *Biomolecular Engineering, 16*, 159.

Wilbur D.S., Chyan M.-K., Hamlin D.K., and Kegley B.B. (2000) Radiolabeled Biotin Reagents for Application to Targeted Therapy of Cancer. Presented at The *First*

International Conference on (Strept)Avidin-Biotin Technologies, held June 18-21, 2000 in Banff, Alberta, Canada. *Biomolecular Engineering, 16*, 162-163.

Wilbur D.S., Chyan M.-K., Hamlin D.K., and Brechbiel M.W. (2000) Synthesis and Radiolabeling of a New Biotin-CHX-A´´ Derivative for Use with In-111, Y-90, or Bi-213 in Tumor Pretargeting. Presented at the 47th Annual Meeting of the Society of Nuclear Medicine, June 3-7 in St. Louis, MO., *J. Nucl. Med. 41*, 120P, 2000.

Wilbur D.S., Hamlin D.K., Chyan M.-K., Kegley B.B., Buhler K.R., Vessella R.L., and Brechbiel M.W. (2000) Optimization of Biotin Derivative Structure to Carry Alpha-Emitting Radionuclides in Pretargeting of Cancer. To be presented at the Symposium on "Radionuclides for Therapeutic Oncology" , Pacifichem 2000 meeting, December 14-19, Honolulu, Hawaii.

Manuscript: (In Preparation)

Wilbur D.S., Chyan M.-K., Hamlin D.K., Kegley B.B., Quinn J., Vessella R.L., and Brechbiel M. (2001) Biotin Reagents in Antibody Pretargeting. 7. Synthesis, Radiolabeling and Evaluation of In-111, Y-90, and Bi-213 labeled Biotin-CHX-A´´ and Biotin-DOTA Derivatives. *Bioconjugate Chemistry – in preparation.*

E. CONCLUSIONS:

At the time of writing the proposal for these studies we were optimistic that they could be completed as outlined. However, during the course of our studies it became apparent that additional tasks were required that had not been planned for. In addition to this, difficulties with obtaining an antibody to conduct the studies set some of the experiments back several months. These factors led to our not completing the tasks originally outlined. However, very valuable information was obtained that will help us push forward with studies of the use of the alpha-emitting radionuclide Bi-213 to treat metastatic cancer. The studies have provided valuable information on the reagents that can be used to target Bi-213 to breast cancer cells. We believe that we have developed the biotin derivative that can be successfully used in vivo for carrying Bi-213. The studies have also shown that Bi-213 labeled succinylated streptavidin is probably not a good choice for further evaluation. Importantly, the studies have helped us to understand the weaknesses in the system that we are trying to develop, and will direct our efforts towards important studies that are required to bring the development to a stage of studying therapeutic efficacy in an animal model.

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G. APPENDICES:

Appendix Item 1: Letter from Dr. Ceriani assuring us that we will get huBrE-3 for the studies

Appendix Item 2: Graphs of results obtained using different methods of measurement of the MCF-7 subrenal capsule size.

Appendix Item 3: Cell Survival data for external beam irradiation, irradiation with low and high specific activity ^{213}Bi-labeled antibody, and ^{213}Bi-labeled non-specific antibody.

Appendix Item 4: List of personnel that spent effort on the studies conducted.

APPENDIX ITEM 1

APPENDIX ITEM 1

Cancer Research Institute of Contra Costa
"great oaks from little acorns grow"

June 12, 1997

Dr. Scott Wilbur
Department of Radiation Oncology
Radiochemistry Group
University of Washington
2121 N. 35th Street
Seattle, WA 98103-9103

Dear Dr. Wilbur:

In response to your letter of June 3, 1997, and its contents, I wish to assure my supply of antibody huBrE-3 (up to 100mg) to be used in your project "Preclinical Evaluation of a Targeted Alpha-Emitting Radionuclide in Radiotherapy of Breast Cancer" in accordance with the conditions of your request. This antibody cannot be used for any commercial purpose and cannot be administered to humans.

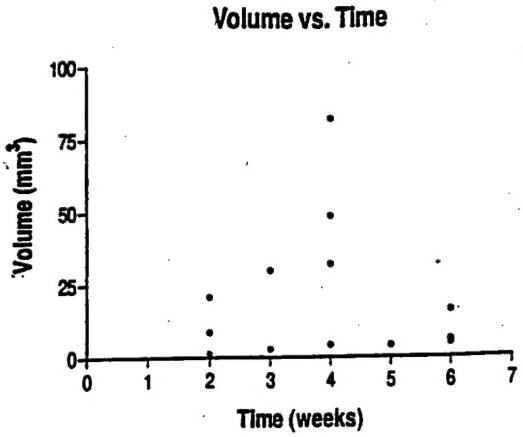
I believe your project proposes a very interesting new use for our antibody and I look forward to a successful collaboration.

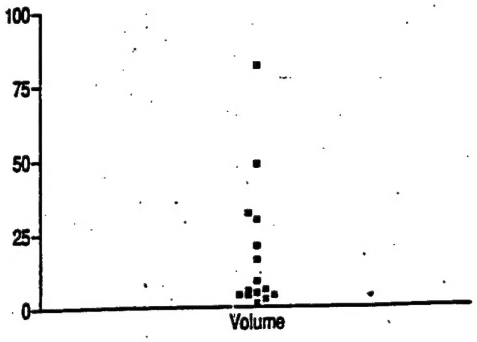
Sincerely,

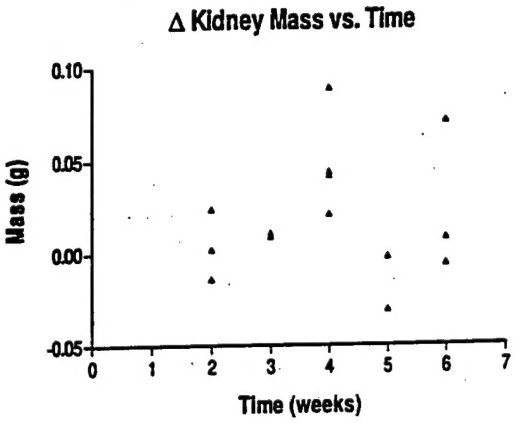
Roberto L. Ceriani, M.D., Ph.D.
Principal Investigator
RLC/jjdb
rle 5/a:\103.ltr

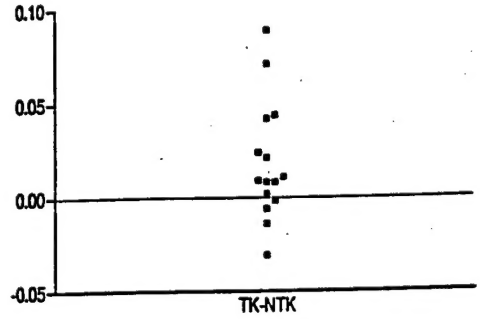
2055 North Broadway • Walnut Creek, CA 94596 • (510) 943-1167 • Fax (510) [illegible]

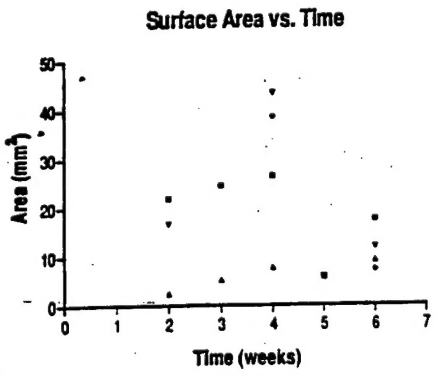
Appendix Item 2

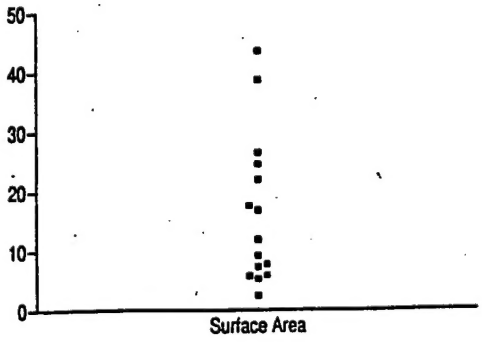




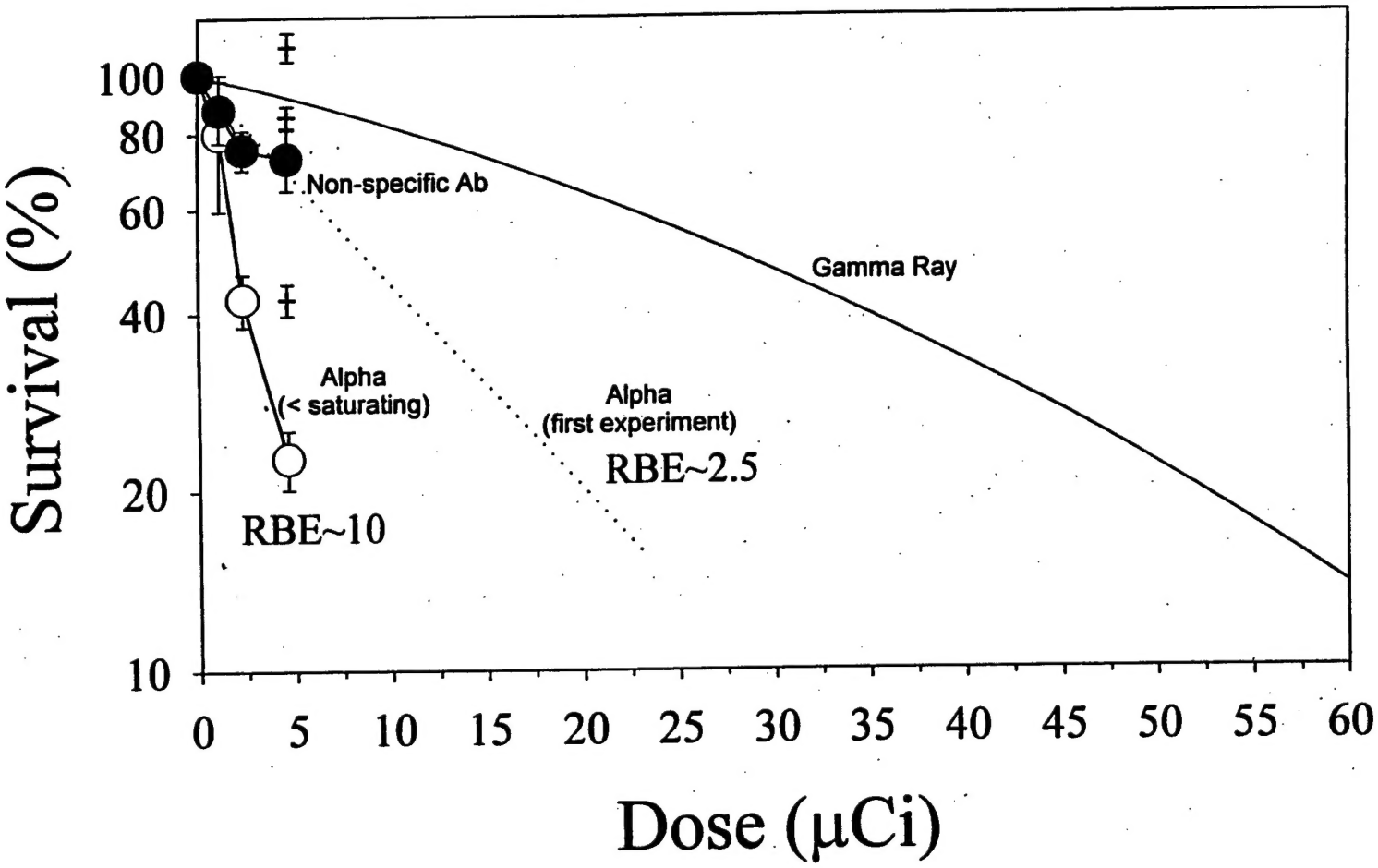








APPENDIX ITEM 3



APPENDIX ITEM 4

PERSONNEL INVOLVED WITH STUDIES (THOSE RECEIVING PAY)

DEPARTMENT OF RADIATION ONCOLOGY (Chemistry and Radiolabeling)

Dr. D. Scott Wilbur (P.I.)

Dr. Jeffrey Schwartz (Investigator)

Dr. Feng Wan (Postdoctoral Fellow)

Mr. Don Hamlin (Research Scientist)

DEPARTMENT OF UROLOGY (Animal Studies and Cell Culture)

Mr. Kent Buhler (Research Scientist)

Ms. Lisha Brown (Research Scientist)

Ms. Janna Quinn (Research Scientist)

Ms. Sarah Whitney (Research Technician)

Mr. Austin Odman (Research Technician)